

# Tissue-Specific Sequence Alterations in the Human Immunodeficiency Virus Type 1 Envelope Favoring CCR5 Usage Contribute to Persistence of Dual-Tropic Virus in the Brain<sup>▽</sup>

Lachlan Gray,<sup>1,2,†</sup> Michael Roche,<sup>1,3,†</sup> Melissa J. Churchill,<sup>1,3</sup> Jasminka Sterjovski,<sup>1,3</sup> Anne Ellett,<sup>1</sup> Pantelis Poubourios,<sup>1</sup> Shameem Sheffief,<sup>4</sup> Bin Wang,<sup>4</sup> Nitin Saxena,<sup>4</sup> Damian F. J. Purcell,<sup>2</sup> Steven Wesselingh,<sup>2,3</sup> Anthony L. Cunningham,<sup>4</sup> Bruce J. Brew,<sup>5</sup> Dana Gabuzda,<sup>6,7</sup> and Paul R. Gorry<sup>1,2,3\*</sup>

*Macfarlane Burnet Institute for Medical Research and Public Health, Melbourne, Victoria, Australia<sup>1</sup>; Department of Microbiology and Immunology, University of Melbourne, Melbourne, Victoria, Australia<sup>2</sup>; Department of Medicine, Monash University, Melbourne, Victoria, Australia<sup>3</sup>; Westmead Millennium Institute, Westmead, New South Wales, Australia<sup>4</sup>; Department of Neurology and St. Vincent's Centre for Applied Medical Research, St. Vincent's Hospital, Darlinghurst, New South Wales, Australia<sup>5</sup>; Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, Boston, Massachusetts<sup>6</sup>; and Department of Neurology, Harvard Medical School, Boston, Massachusetts<sup>7</sup>*

Received 23 December 2008/Accepted 17 March 2009

Most human immunodeficiency virus type 1 (HIV-1) strains isolated from the brain use CCR5 for entry into macrophages and microglia. Strains that use both CCR5 and CXCR4 for entry (R5X4 strains) have been identified in the brains of some individuals, but mechanisms underlying the persistence of R5X4 viruses compartmentalized between the brain and other tissue reservoirs are unknown. Here, we characterized changes in the HIV-1 envelope (Env) that enhance the tropism of R5X4 variants for brain or lymphoid tissue. R5X4 Envs derived from the brains of two individuals had enhanced CCR5 usage in fusion assays compared to R5X4 Envs derived from matched spleen or blood, which was associated with reduced dependence on specific residues in the CCR5 N terminus and extracellular loop 1 (ECL1) and ECL3 regions. In contrast, spleen/blood-derived Envs had enhanced CXCR4 usage compared to brain-derived Envs, which was associated with reduced dependence on residues in the CXCR4 N terminus and ECL2 region. Consequently, brain-derived Envs had preferential CCR5 usage for HIV-1 entry into the JC53 cell line, could use either CCR5 or CXCR4 for entry into monocyte-derived macrophages (MDM), and could use CCR5 (albeit inefficiently) for entry into peripheral blood mononuclear cells (PBMC), whereas the entry of spleen-derived Envs was CXCR4 dependent in all three cell types. Mutagenesis studies of Env amino acid variants influencing coreceptor usage showed that S306 in the gp120 V3 region of brain-derived Envs reduces dependence on the CCR5 N terminus and enhances CCR5 usage for HIV-1 entry into PBMC and MDM, whereas R306 in spleen-derived Envs reduces dependence on the CXCR4 N terminus and confers the CXCR4 restricted phenotype. These results identify mechanisms underlying R5X4 HIV-1 persistence in different tissue reservoirs. Tissue-specific changes in the gp120 V3 region that increase the efficiency of CCR5 or CXCR4 usage, and thereby influence coreceptor preference, may enhance the tropism of R5X4 strains for CCR5-expressing macrophage lineage cells in the brain and CXCR4-expressing T cells in lymphoid tissues, respectively.

Human immunodeficiency virus type 1 (HIV-1) establishes infection in central nervous system (CNS) and peripheral tissues, which poses barriers to effectively treating HIV-1 infection (2, 16). Resting memory CD4<sup>+</sup> T cells in blood, spleen, and lymph nodes (62) and CD16<sup>+</sup> monocytes in blood (18) are significant latently infected reservoirs of HIV-1 that prevent eradication of HIV-1 by highly active antiretroviral therapy. Microglia and macrophages are target cells for HIV-1 replication in the brain (reviewed in references 16 and 29). They are less susceptible to the cytopathic effects of HIV-1 than acti-

vated CD4<sup>+</sup> T cells (27, 28), so they may continue to shed virus for the duration of their normal life spans. Since most antiretroviral therapies have poor CNS penetration (52, 63), the brain is a significant reservoir for viral persistence.

The genetic evolution of HIV-1 within the brain is distinct from that in lymphoid tissues and other organs (references 30 and 33 and references therein). Specific sequences within the viral envelope glycoprotein (Env), particularly the gp120 V3 region, have been associated with brain infection (reviewed in reference 16). The genetic compartmentalization of viral variants in the CNS suggests that adaptive changes may occur in response to unique constraints of the CNS microenvironment, such as different target cell populations and immune selection pressures.

The HIV-1 Env protein is organized into trimers on virions and consists of the gp120 surface and gp41 transmembrane subunits. HIV-1 entry into cells is initiated by a high-affinity

\* Corresponding author. Mailing address: Macfarlane Burnet Institute for Medical Research and Public Health, 85 Commercial Rd., Melbourne, 3004, Victoria, Australia. Phone: 61-3-9282-2129. Fax: 61-3-9282-2100. E-mail: gorry@burnet.edu.au.

† L.G. and M.R. contributed equally.

<sup>▽</sup> Published ahead of print on 25 March 2009.

interaction between gp120 and CD4, which induces a conformational change in gp120 that exposes the binding site for a chemokine coreceptor, which is either of the seven transmembrane-spanning G protein-coupled receptors CCR5 and CXCR4 (11, 12). Current models of gp120 binding to the coreceptor suggest the gp120 V3 loop interacts principally with the coreceptor second extracellular loop (ECL2) region, while the gp120 bridging sheet formed between the C1, C2, and C4 domains of gp120 after CD4 binding interacts with the coreceptor N terminus (4, 8, 21, 38). There may be functional redundancy for the coreceptor N terminus if the V3 loop has an enhanced interaction with the coreceptor ECL2 region (43). The V3 loop of gp120 is the primary determinant of coreceptor specificity (5, 37), and bioinformatics algorithms based on the V3 amino acid sequence have been developed to predict whether HIV-1 uses CCR5 or CXCR4 for virus entry (39, 57, 70). While the coreceptor N terminus and ECL2 region appear to be important for gp120-coreceptor binding, the ECL1 and ECL3 regions may also influence the coreceptor function of G protein-coupled receptors (13, 14, 20). The interaction of CD4-bound gp120 with the coreceptor induces additional conformational changes in gp120, which leads to a structural rearrangement in gp41 that enables fusion and virus entry.

The tropism of HIV-1 for particular target cell populations in different tissue compartments is influenced by the coreceptor used by HIV-1 Env for virus entry (16, 31). Macrophage-tropic HIV-1 primarily uses CCR5 (R5) as a coreceptor (1, 6, 15), whereas T-cell line-tropic viruses use CXCR4 (X4) (23). Dual-tropic viruses can use both coreceptors (R5X4) (7, 73). However, while R5X4 viruses isolated from blood may use either CCR5 or CXCR4 for HIV-1 entry into macrophages (73), they principally use CXCR4 for HIV-1 entry into CD4<sup>+</sup> T cells (74).

CCR5 is the principal coreceptor for HIV-1 infection of macrophages and microglia and is the coreceptor used by most viruses isolated from the brain and cerebrospinal fluid (CSF) [(references 30 and 33 and references therein). Only three cases in which R5X4 HIV-1 has been isolated from the brain have been reported (30, 33, 48, 50), suggesting that persistence of R5X4 HIV-1 in the brain is rare. However, many brain-derived HIV-1 strains have been characterized by V3 sequence rather than traditional phenotypic assays (10, 42, 47, 56, 59–61, 64). Recent studies suggest V3 sequence-based algorithms may underestimate the frequency of CXCR4 usage by R5X4 HIV-1 strains (46, 48), and in fact, the reported brain-derived HIV-1 strains that have the R5X4 phenotype in HIV-1 entry assays are scored as “R5-like” viruses using these algorithms (48). Therefore, it is likely that the frequency of R5X4 HIV-1 variants in the brain has been underestimated. Consequently, mechanisms underlying the persistence of R5X4 viruses compartmentalized between the brain and other tissue reservoirs are poorly understood. In this study, we characterized changes in gp120 contributing to compartmentalization of R5X4 Envs between the brain and matched lymphoid tissues.

#### MATERIALS AND METHODS

**Cells.** Peripheral blood mononuclear cells (PBMC) were purified from the blood of healthy HIV-1-negative donors, stimulated with 5 µg of phytohemagglutinin (Sigma, St. Louis, MO) per ml for 3 days, and cultured in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum (FCS), 100 µg of

penicillin and streptomycin per ml, and 20 U of interleukin-2 (Roche, Basel, Switzerland) per ml. Monocyte-derived macrophages (MDM) were produced from elutriated monocytes that were cultured for 5 days in RPMI 1640 medium supplemented with 10% (vol/vol) pooled human sera, 100 µg of penicillin and streptomycin per ml, and 12.5 ng of macrophage colony-stimulating factor per ml. Cf2-Luc cells (19), derived from the Cf2th canine thymocyte cell line (6) and stably expressing the luciferase gene under the control of the HIV-1 long terminal repeat, were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% (vol/vol) FCS, 100 µg of penicillin and streptomycin per ml, and 0.7 mg of G418 per ml. Cf2-CD4/CCR5 cells (71) were cultured in DMEM supplemented with 10% (vol/vol) FCS, 100 µg of penicillin and streptomycin per ml, 0.5 mg of G418 per ml, and 0.1 mg of hygromycin per ml. Cf2-CD4/CXCR4 cells (65) were cultured in DMEM supplemented with 10% (vol/vol) FCS, 100 µg of penicillin and streptomycin per ml, 0.5 mg of G418 per ml, and 1 µg of puromycin per ml. JC53 cells, derived from the HeLa cell line and stably expressing high levels of CD4, CXCR4, and CCR5 on the cell surface (55), were cultured in DMEM supplemented with 10% (vol/vol) FCS and 100 µg of penicillin and streptomycin per ml. 293T cells were cultured in DMEM supplemented with 10% (vol/vol) FCS and 100 µg of penicillin and streptomycin per ml.

**Primary HIV-1 isolates and Env clones.** The primary R5X4 HIV-1 isolates Macs1-BR and Macs1-SPLN were isolated from autopsy brain and spleen tissue samples from an AIDS patient with dementia and HIV-1 encephalitis by coculture with CD8-depleted PBMC. These viruses have been described in detail previously (30, 33). Full-length primary R5X4 HIV-1 Envs cloned from brain biopsy tissue or blood (aBR01, aBL01, dBR02, and dBR07) have been described previously (50).

**PCR amplification, HIV-1 Env cloning, identification of functional Envs, and sequence analysis.** Viral RNA was purified from Macs1-BR and Macs1-SPLN isolates using a Qiaamp UltraSens Virus purification kit (Qiagen) according to the manufacturers' protocol. cDNA was generated using SuperScript III reverse transcriptase (Invitrogen) and random hexamers according to the manufacturers' protocol. An approximately 2.1-kb fragment spanning the KpnI-BamHI restriction sites in HIV-1 *env* (corresponding to nucleotides 6348 to 8478 in HXB2) was amplified from cDNA by PCR using nested primers and Expand high-fidelity DNA polymerase (Roche Diagnostics), as described previously (34, 50, 65). The outer primers were env1A and env1M (26), and the inner primers were Env-KpnI and Env-BamHI (34, 50). PCR cycling consisted of an initial denaturation step at 94°C for 2 min, followed by 9 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 2 min and then a further 20 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 2 min, but with a 5-s increasing extension time for each cycle, followed by a final extension at 72°C for 7 min. The products of three independent PCRs were purified, pooled, and then cloned into the pSVIII-HXB2 Env expression plasmid (26) by replacement of the 2.1-kb KpnI-BamHI HXB2 *env* fragment. Thus, the resulting Env clones contained the entire gp160 coding region of primary virus-derived *env* genes, except for 36 amino acids at the N terminus and 105 amino acids at the C terminus, which were derived from HXB2. Six functional Env clones from each virus were identified by the ability to support entry when pseudotyped onto Env-deficient green fluorescent protein (GFP) reporter viruses and used in single-round entry assays in JC53 cells, as described previously (65, 67). The coreceptor usage of functional Env clones was verified by single-round entry assays in Cf2-CD4/CCR5 and Cf2-CD4/CXCR4 cells infected with Env-pseudotyped GFP reporter viruses, as described previously (34, 65). The Envs were sequenced by Big Dye terminator sequencing (Applied Biosystems) and analyzed using a model 3100 Genetic Analyzer (Applied Biosystems).

**Env mutagenesis.** Mutagenesis to exchange amino acids at positions 306 and/or 369 in gp120 of Macs1-BR-3 and Macs1-SPLN-12 Envs was performed using the QuikChange II site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The primers used in the mutagenesis studies are available upon request. Env mutants were sequenced in their entirety to confirm the presence of the mutated residue.

**Fusion assays.** Fusion assays were conducted as described previously (17, 32, 33, 65, 67). Briefly, 293T effector cells cotransfected with 3.4 µg of Env-expressing plasmid and 0.6 µg pSVL-Tat plasmid using Lipofectamine 2000 (Invitrogen) were mixed with Cf2-Luc target cells that had been cotransfected with 2 µg of pcDNA3-CD4 and 6 µg of pcDNA3-CCR5 or pcDNA3-CXCR4 and incubated at 37°C in replicate wells of 96-well tissue culture plates containing 200 µl of culture medium. Cells from replicate wells were harvested at 2, 4, 6, 8, 10, and 12 h postmixing and assayed for luciferase activity (Promega) according to the manufacturers' protocol. 293T cells transfected with pSVL-Tat alone were used as negative controls to determine the background level of luciferase activity, which was subtracted from fusion assays. In some experiments, plasmids expressing CCR5 mutants (Y10A, D11A, Y14A, Y15A, H88A, Y89A, H181A, Y184A, Q188A, K197A, E262A, and F264A) or CXCR4 mutants (Δ4-36, R183A,

D193A, and F199A) with attenuated coreceptor activity, which have been described previously (4, 13, 20), were used. In experiments with coreceptor mutants, the 10-h time point was used for analysis. To control for cell surface Env expression levels in 293T effector cells, Env expression was measured by flow cytometry using pooled AIDS serum and a fluorescein isothiocyanate-conjugated anti-human F(ab')<sub>2</sub> immunoglobulin (Chemicon), as described previously (65).

**Production and quantitation of Env-pseudotyped luciferase reporter viruses.** Env-pseudotyped luciferase reporter viruses were produced by transfection of 293T cells with pCMVΔP1ΔenvA, pHIV-1Luc, and pSVIII-Env plasmids using Lipofectamine 2000 (Invitrogen) at a ratio of 1:3:1, as described previously (34, 65, 72). The supernatants were harvested 48 h later, filtered through 0.45-μm filters, and stored at -80°C. The 50% tissue culture infective doses (TCID<sub>50</sub>) of virus stocks were determined by titration in JC53 cells.

**HIV-1 entry assays.** Ten thousand JC53 cells were inoculated with 100 TCID<sub>50</sub> of Env-pseudotyped luciferase reporter virus (equating to a multiplicity of infection [MOI] of 0.01) in a volume of 100 μl for 12 h at 37°C. The cells were washed twice with culture medium to remove residual inoculum and incubated for a further 72 h at 37°C. Two hundred thousand PBMC were inoculated with 2,000 TCID<sub>50</sub> of Env-pseudotyped luciferase reporter virus (equating to an MOI of 0.01) in a volume of 100 μl for 12 h at 37°C. The cells were washed twice with culture medium to remove residual inoculum and incubated for a further 72 h at 37°C. Fifty thousand MDM were inoculated with 2,000 TCID<sub>50</sub> of Env-pseudotyped luciferase reporter virus (equating to an MOI of 0.04) in a volume of 100 μl for 12 h at 37°C. The cells were washed twice with culture medium to remove residual inoculum and incubated for a further 96 h at 37°C. In experiments measuring the inhibition of HIV-1 entry by maraviroc and/or AMD3100, cells were either left untreated or preincubated for 30 min with 1 μM of each inhibitor prior to inoculation with virus. In drug-treated cells, the drug concentrations were maintained during virus inoculation and the subsequent culture period. At the end of the culture period, HIV-1 entry was measured by assaying luciferase activity in cell lysates (Promega), according to the manufacturer's protocol. Negative controls included mock-infected cells that were incubated with culture medium instead of virus and cells inoculated with luciferase reporter virus pseudotyped with the nonfunctional ΔKS Env (19). After the background luciferase activity was subtracted, the amount of luciferase activity in cells treated with maraviroc and/or AMD3100 was expressed as a percentage of that in untreated cells. In studies using HIV-1 entry assays, two independent preparations of reporter virus were used.

**Nucleotide sequence accession numbers.** The Macs1-BR and Macs1-SPLN Env nucleotide sequences reported here have been assigned GenBank accession numbers FJ687532 to FJ687543.

## RESULTS

**Functional R5X4 HIV-1 Env clones derived from the brain and other tissues.** We previously identified R5X4 HIV-1 from the brains of three subjects with HIV-associated dementia (HAD) (30, 33, 48, 50). Primary HIV-1 isolates were derived from autopsy specimens of the brain and spleen of subject Macs1 (Macs1-BR and Macs1-SPLN, respectively) and were R5X4 phenotype in Cf2-Luc cells expressing CD4 and CCR5 or CXCR4 (30). Full-length Envs were cloned directly from the brain biopsy tissue or blood of subject A (aBR01 and aBL01, respectively) and from brain biopsy tissue of subject D (dBR02 and dBR07) (50) and were R5X4 phenotype in fusion assays and single-round entry assays, although aBR01 Env used CXCR4 weakly compared to CCR5 (50). No functional Envs were obtained from the blood of subject D. There are no other reported cases of R5X4 HIV-1 isolated from the brain (48).

To investigate the mechanisms underlying R5X4 HIV-1 persistence in different tissue compartments, the gp160 coding region of Macs1-BR and Macs1-SPLN viruses was cloned into the pSVIII-Env expression plasmid (26) for comparison with the phenotype of the aBR01 and aBL01 Envs. Six functional Env clones from each virus were identified based on the ability to support entry of Env-pseudotyped GFP reporter viruses into

CD4/CXCR4/CCR5-expressing JC53 cells (data not shown). These Envs, referred to hereafter as Macs1-BR-2, -3, -4, -5, -6, and -8 and Macs1-SPLN-3, -4, -7, -10, -12, and -15, were further characterized for the ability to use CCR5 or CXCR4 for entry into Cf2th cells expressing CD4 and either coreceptor as described previously (65). Reporter viruses pseudotyped with R5 ADA, X4 HXB2, or R5X4 89.6 Env were used as controls and entered cells expressing CCR5, CXCR4, or both CCR5 and CXCR4, respectively (data not shown). All Envs from Macs1-BR and Macs1-SPLN used both CCR5 and CXCR4 for virus entry, indicating an R5X4 phenotype (data not shown). Thus, we established a panel of functional R5X4 Envs from the known cases of brain R5X4 HIV-1 infection and, where possible, from matched lymphoid tissue or blood.

**Coreceptor-dependent fusion of R5X4 Envs.** A quantitative cell-cell fusion assay was used to compare the abilities of brain- and spleen/blood-derived R5X4 Envs to mediate CCR5- or CXCR4-dependent fusion (Fig. 1). Compared to spleen/blood-derived Envs, Macs1-BR and aBR01 Envs mediated greater levels of fusion in cells expressing CD4 and CCR5 (Fig. 1A). Conversely, compared to brain-derived Envs, Macs1-SPLN and aBL01 Envs mediated greater levels of fusion in cells expressing CD4 and CXCR4 (Fig. 1B). Similar levels of gp120 were detected on the surfaces of effector cells by flow cytometry (data not shown). These data indicate greater CCR5-dependent fusogenicity by the brain-derived R5X4 Envs and greater CXCR4-dependent fusogenicity by the spleen/blood-derived R5X4 Envs.

**Coreceptor preference of R5X4 Envs for HIV-1 entry.** To determine whether R5X4 Envs from different tissue compartments have altered coreceptor preferences for HIV-1 entry, luciferase reporter viruses pseudotyped with Macs1-BR and Macs1-SPLN Envs were produced and used to inoculate JC53 cells that were treated with inhibitory concentrations of the CCR5 inhibitor maraviroc, the CXCR4 inhibitor AMD3100, or a combination of both inhibitors. Luciferase reporter viruses pseudotyped with ADA, HXB2, or 89.6 Env were used as controls (Fig. 2A). The entry of viruses pseudotyped with Macs1-SPLN Envs was almost completely inhibited by AMD3100, similar to virus pseudotyped with the 89.6 Env, but was only marginally inhibited by maraviroc (Fig. 2B). In contrast, the entry of viruses pseudotyped with Macs1-BR Envs was more potently inhibited by maraviroc than by AMD3100. The combination of both compounds completely inhibited the entry of all viruses. Thus, Macs1-SPLN Envs use CXCR4 almost exclusively for HIV-1 entry into JC53 cells, whereas Macs1-BR Envs can use either coreceptor for HIV-1 entry but have a preference for CCR5.

**Dependence of R5X4 Envs on N-terminal and ECL regions of CCR5 and CXCR4.** To elucidate differences in the mechanisms of coreceptor usage by the brain- and spleen/blood-derived R5X4 Envs, fusion assays were conducted with target cells expressing CD4 and a mutant coreceptor containing amino acid alterations in the N terminus or ECL1, ECL2, or ECL3 region of CCR5 or CXCR4 that attenuated coreceptor activity (Fig. 3). Compared to Macs1-SPLN and aBL01 Envs, Macs1-BR and aBR01 Envs mediated greater levels of fusion in cells expressing CCR5 with mutations in the N-terminal (Y15A), ECL1 (H88A), and ECL3 (E262A and F264A) regions (Fig. 3A and C). Compared to Macs1-BR and aBR01 Envs, Macs1-SPLN and aBL01 Envs mediated greater levels of



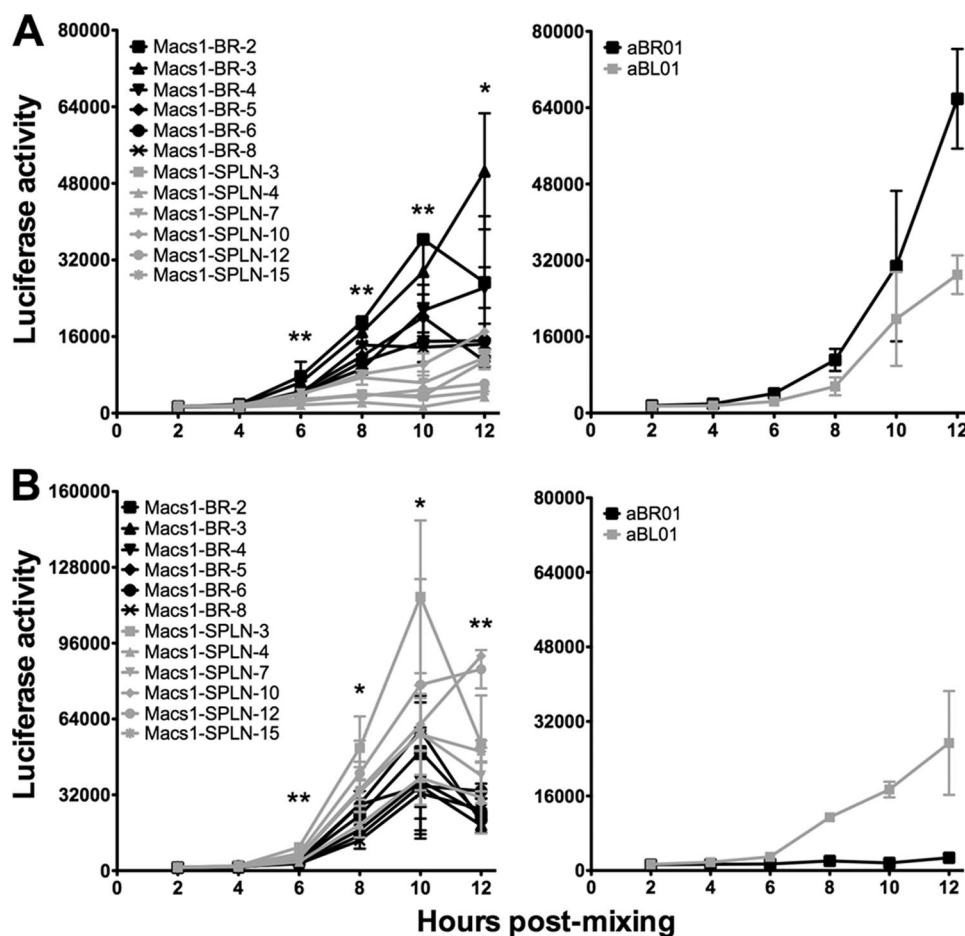


FIG. 1. CCR5- and CXCR4-dependent fusion of brain- and spleen/blood-derived R5X4 Envs. Fusion assays were performed using 293T effector cells expressing Macs1-BR or Macs1-SPLN Envs (left) or aBR01 or aBL01 Envs (right) and Cf2-Luc target cells expressing CD4 and CCR5 (A) or CXCR4 (B), as described in Materials and Methods. The data shown are representative of three independent experiments and are expressed as mean values from duplicate wells; the error bars represent standard deviations. *P* values were calculated using a nonparametric Mann-Whitney U test; \*, *P* < 0.05; \*\*, *P* < 0.01.

fusion in cells expressing CXCR4 with a large deletion in the N terminus ( $\Delta 4-36$ ) and with a mutation in the ECL2 region (R183A) (Fig. 3B and D). There were no significant differences in levels of fusion between brain- and spleen/blood-derived Envs in cells expressing CCR5 with mutations in the ECL2 region (H181A, Y184A, Q188A, and K197A), in cells expressing CCR5 with other mutations in the N-terminal (Y10A, D11A, and Y14A) and ECL1 (Y89A) regions, and in cells expressing CXCR4 with other mutations in the ECL2 (D193A and F199A) region (data not shown). Similar levels of gp120 were detected on the surfaces of effector cells by flow cytometry (data not shown). Thus, brain-derived R5X4 Envs from both subjects had reduced dependence on specific residues in the CCR5 N terminus and ECL1 and ECL3 regions for CCR5-dependent fusion, suggesting more efficient CCR5 usage. In addition, the spleen/blood-derived R5X4 Envs from both subjects had reduced dependence on the CXCR4 N terminus and R183 in the ECL2 region for CXCR4-dependent fusion, suggesting more efficient CXCR4 usage.

**Analysis of HIV envelope sequence.** To investigate the mechanisms underlying the divergent patterns of CCR5 and

CXCR4 usage by brain- and spleen-derived R5X4 Envs, respectively, the gp120 regions of the Macs1-BR and Macs1-SPLN clones were sequenced and analyzed to identify amino acid variants that might influence coreceptor usage. Amino acid alignments of gp120 showed that all of the Envs were unique, and phylogenetic analysis showed distinct clustering between brain- and spleen-derived sequences (data not shown). Two amino acid changes distinguished brain- and spleen-derived Env sequences (Fig. 4A). Serine was present at position 369 within a CD4 binding domain in the C3 regions of six of six Macs1-SPLN Envs, whereas proline was present at this position in six of six Macs1-BR Envs and 89.6 R5X4 Env, which may influence the efficiency of CD4 binding. Arginine was present at position 306 in the V3 loops of six of six Macs1-SPLN Envs and 89.6 Env, whereas serine was present at this position in six of six Macs1-BR Envs. Amino acid 306 was in position 11 in the V3 loop of gp120; the presence of a basic amino acid at this position or at position 25 in V3 is a predictor of CXCR4 usage (39, 57). Since the Macs1-BR Envs lack basic amino acids at either position, bioinformatics prediction programs score these Envs as “R5-like” despite being functionally

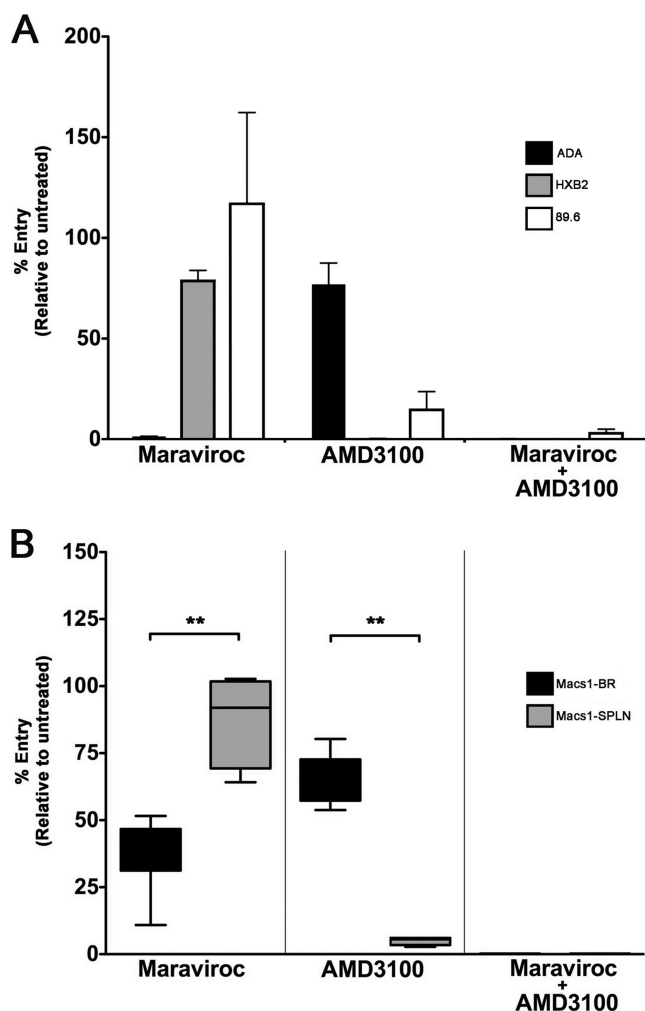


FIG. 2. Coreceptor preference for HIV-1 entry into JC53 cells. JC53 cells were treated with maraviroc (1  $\mu$ M), AMD3100 (1  $\mu$ M), or both inhibitors prior to infection with equivalent infectious units of Env-pseudotyped luciferase reporter virus, as described in Materials and Methods. Untreated cells contained no inhibitor. HIV-1 entry was measured by luciferase activity in cell lysates and expressed as a percentage of entry in untreated cells. (A) Inhibition of HIV-1 entry in cells inoculated with luciferase reporter virus pseudotyped with ADA, HXB2, or 89.6 Env. The data are expressed as mean values from triplicate infections, and the error bars represent standard deviations. (B) Inhibition of HIV-1 entry in cells inoculated with luciferase reporter virus pseudotyped with Macs1-BR Envs (clones Macs1-BR-2, -3, -4, -5, -6, and -8) or Macs1-SPLN Envs (clones Macs1-SPLN-3, -7, -10, and -12). The titers of luciferase reporter viruses pseudotyped with Macs1-SPLN-4 and -15 Envs were not sufficient for these experiments and subsequent entry assays. Box plots were constructed from the mean values of triplicate infections with each virus using Prism version 5.0a (GraphPad Software, San Diego, CA.). The boxes represent upper and lower quartiles and median scores, and the whiskers represent minimum and maximum values. The results are representative of three independent experiments. *P* values were calculated using a nonparametric Mann-Whitney U test; \*\*, *P* < 0.01.

R5X4 (48). These results suggest that the lack of a basic amino acid at position 306 in the V3 loop region of Macs1-BR Envs may favor CCR5 usage.

**gp120 amino acid variants influencing coreceptor preference.** Mutagenesis was performed to determine the influence

of amino acid variations at positions 306 and 369 on preferential CCR5 and CXCR4 usage by brain- and spleen-derived R5X4 Envs, respectively, for HIV-1 entry into JC53 cells. The S306R mutation in Macs1-BR-3 Env rendered luciferase reporter virus completely resistant to inhibition by maraviroc and almost completely sensitive to inhibition by AMD3100, similar to Macs1-SPLN-12 (Fig. 4B) and 89.6 Envs (Fig. 2A). The R306S mutation in Macs1-SPLN-12 Env rendered luciferase reporter virus partially sensitive to inhibition by either maraviroc or AMD3100, similar to Macs1-BR-3 Env. Additional mutagenesis studies showed that P369 and S369 had no effect on coreceptor preference when these amino acids were exchanged either in isolation or in combination with S306R or R306S Env mutations (data not shown). Thus, R306 in gp120 confers preferential CXCR4 usage by Macs1-SPLN Envs for HIV-1 entry into JC53 cells, whereas S306 (or the lack of a basic amino acid at this position) enables Macs1-BR Envs to use either CCR5 or CXCR4 for HIV-1 entry.

**V3 loop alterations influence dependence of R5X4 Envs on the CCR5 or CXCR4 N terminus.** To determine the influence of S306 in Macs1-BR Envs or R306 in Macs1-SPLN Envs on reduced dependence on N-terminal and ECL regions of CCR5 and CXCR4, respectively, parental and mutant Envs were used in fusion assays with cells expressing wild-type or mutant coreceptors (Fig. 5). Compared to parental Envs, the S306R mutation in Macs1-BR-3 Env caused an approximately twofold reduction in fusion levels in cells expressing the CCR5 Y15A mutant, whereas the R306S mutation in Macs1-SPLN-12 Env caused an approximately sixfold increase in fusion levels (Fig. 5A). However, compared to parental Envs, the S306R mutation in Macs1-BR-3 Env caused an approximately sixfold increase in fusion levels in cells expressing the CXCR4  $\Delta$ 4-36 mutant, whereas the R306S mutation in Macs1-SPLN-12 Env caused an approximately fivefold decrease in fusion levels (Fig. 5B). Similar levels of gp120 were detected on the surfaces of effector cells by flow cytometry (data not shown). These results suggest S306 reduces the dependence of brain-derived R5X4 Envs on Y15 in the CCR5 N terminus for CCR5-dependent fusion and that R306 reduces the dependence of spleen-derived R5X4 Envs on the CXCR4 N terminus for CXCR4-dependent fusion. There were no significant differences in levels of fusion between parental and mutant Envs in cells expressing CCR5 H88A, CCR5 E262A, CCR5 F264A, or CXCR4 R183A mutants (data not shown). Together, these data suggest that amino acid variations at position 306 in gp120 influence changes in the dependence of R5X4 Envs on the CCR5 or CXCR4 N terminus but do not influence changes in dependence on residues in the ECL regions.

**V3 loop alterations influence coreceptor utilization of R5X4 Envs for HIV-1 entry into primary cells.** Previous studies showed that most blood-derived R5X4 strains of HIV-1 can use CCR5 or CXCR4 for HIV-1 entry into macrophages, with coreceptor preference dependent on the strain, but are CXCR4 restricted for entry into PBMC (74). Since the brain- and spleen-derived R5X4 Envs have altered coreceptor preferences for HIV-1 entry into JC53 cells (Fig. 2) and have amino acid alterations in the V3 region of gp120 that influence coreceptor preference (Fig. 4), we next determined the coreceptors used by these Envs for HIV-1 entry into PBMC or MDM and the influence of V3 loop alterations on coreceptor

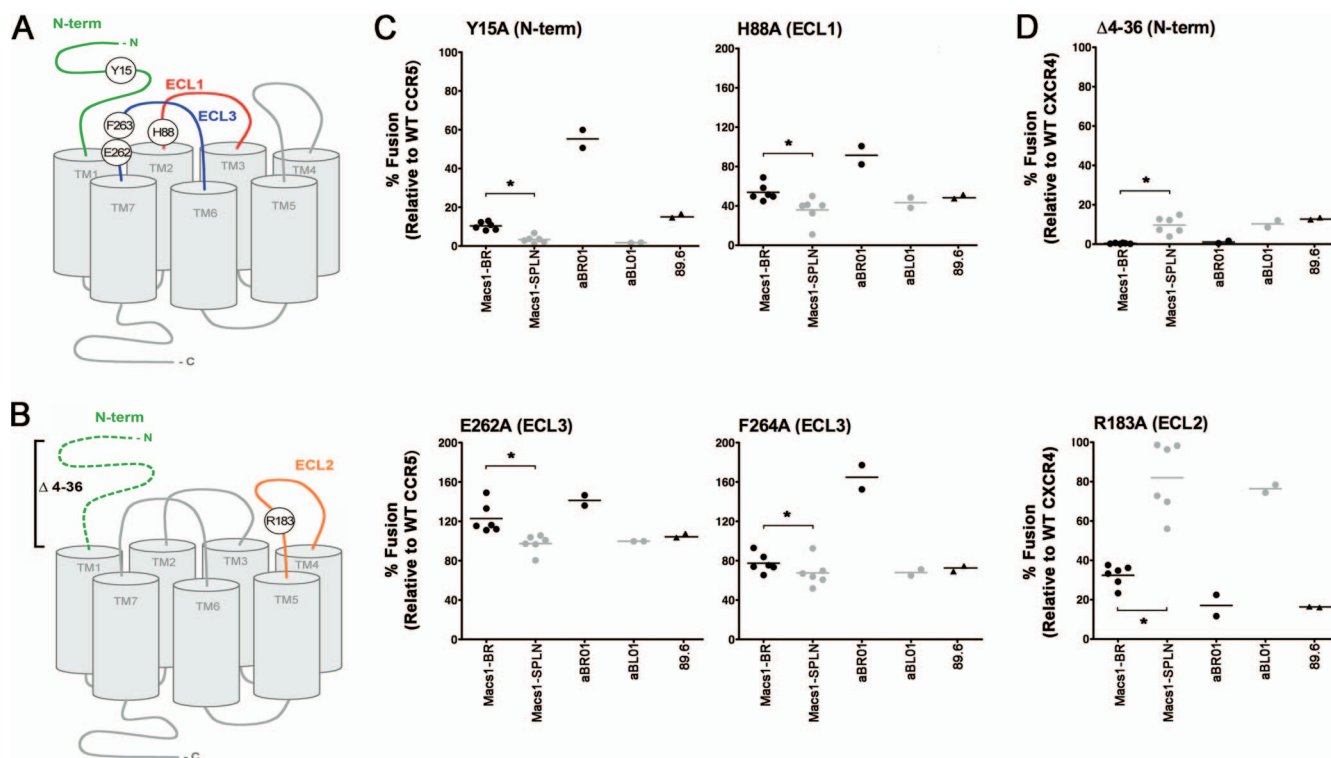


FIG. 3. Dependence on N-terminal and ECL regions of CCR5 and CXCR4. Fusion assays were performed using 293T effector cells expressing Macs1-BR, Macs1-SPLN, aBR01, or aBL01 Env and Cf2-Luc target cells expressing CD4 and either wild-type CCR5 or a CCR5 mutant (C) or either wild-type CXCR4 or a CXCR4 mutant (D), as described in Materials and Methods. 293T cells expressing 89.6 Env were included as controls. Panels A and B illustrate the regions in CCR5 and CXCR4, respectively, where the mutations were present. After the background was subtracted, luciferase activity in fusion assays with mutant coreceptors was expressed as a percentage of that in fusion assays with wild-type coreceptor. Each data point in fusion assays with Macs1-BR and Macs1-SPLN Envs represents the mean value of duplicate wells for each clone. Since only single Env clones were available for aBR01 and aBL01 Envs (and 89.6 Env), each data point in fusion assays with these Envs represents an individual duplicate. The results are representative of four independent experiments. *P* values were calculated using a nonparametric Mann-Whitney U test; \*, *P* < 0.05.

usage. In primary cell experiments, luciferase reporter viruses pseudotyped with ADA, HXB2, or 89.6 Envs were used as controls for entry assays in PBMC (Fig. 6A), and reporter viruses pseudotyped with ADA or 89.6 Envs were used as controls for entry assays in MDM (Fig. 6C).

In PBMC, maraviroc was unable to inhibit the entry of brain- and spleen-derived R5X4 Envs at detectable levels, but AMD3100 inhibited the entry of brain- and spleen-derived R5X4 Envs by approximately 70% and 80%, respectively (Fig. 6B). These results suggest that, unlike JC53 cells, brain-derived R5X4 Envs exhibit preferential CXCR4 usage for entry into PBMC. The combination of both inhibitors was unable to further inhibit the entry of spleen-derived R5X4 Envs, suggesting these Envs are CXCR4 restricted for entry into PBMC, similar to 89.6 Env (Fig. 6A) and primary blood-derived R5X4 HIV-1 strains (74). However, the combination of both agents inhibited the entry of brain-derived R5X4 Envs by an additional 30%, achieving complete inhibition, indicating that brain-derived R5X4 Envs may use CCR5 for entry into PBMC, albeit at lower efficiency than CXCR4.

In MDM, maraviroc was unable to inhibit the entry of spleen-derived R5X4 Envs and inhibited the entry of brain-derived R5X4 Envs to variable levels, but AMD3100 completely inhibited the entry of spleen-derived R5X4 Envs and

inhibited the entry of brain-derived R5X4 Envs by approximately 60% (Fig. 6D). The combination of both inhibitors completely inhibited the entry of brain-derived R5X4 Envs. Thus, as in PBMC, spleen-derived R5X4 Envs are CXCR4 restricted for entry into MDM whereas brain-derived R5X4 Envs may use either CCR5 or CXCR4 for HIV-1 entry.

We next investigated the influence of Ser or Arg at position 306 in gp120 on coreceptor utilization for HIV-1 entry into PBMC or MDM using Env mutants. In PBMC, entry of the Macs1-BR-3 S306R Env mutant was completely inhibited by AMD3100 with no further inhibition by the combination of both AMD3100 and maraviroc, similar to Macs1-SPLN-12 Env (Fig. 7A). Conversely, entry of the Macs1-SPLN-12 R306S Env mutant was incompletely inhibited by AMD3100 and was completely inhibited by the combination of both AMD3100 and maraviroc, similar to Macs1-BR-3 Env. In MDM, entry of the Macs1-BR-3 S306R Env mutant was not inhibited by maraviroc and was completely inhibited by AMD3100, similar to Macs1-SPLN-12 Env (Fig. 7B). Conversely, entry of the Macs1-SPLN-12 R306S Env mutant was partially inhibited by maraviroc or AMD3100 and was completely inhibited by the combination of both maraviroc and AMD3100, similar to Macs1-BR-3 Env. Together, these results indicate R306 confers a CXCR4-restricted phenotype on spleen-derived R5X4 Envs for HIV-1 entry into PBMC and MDM, whereas S306 in

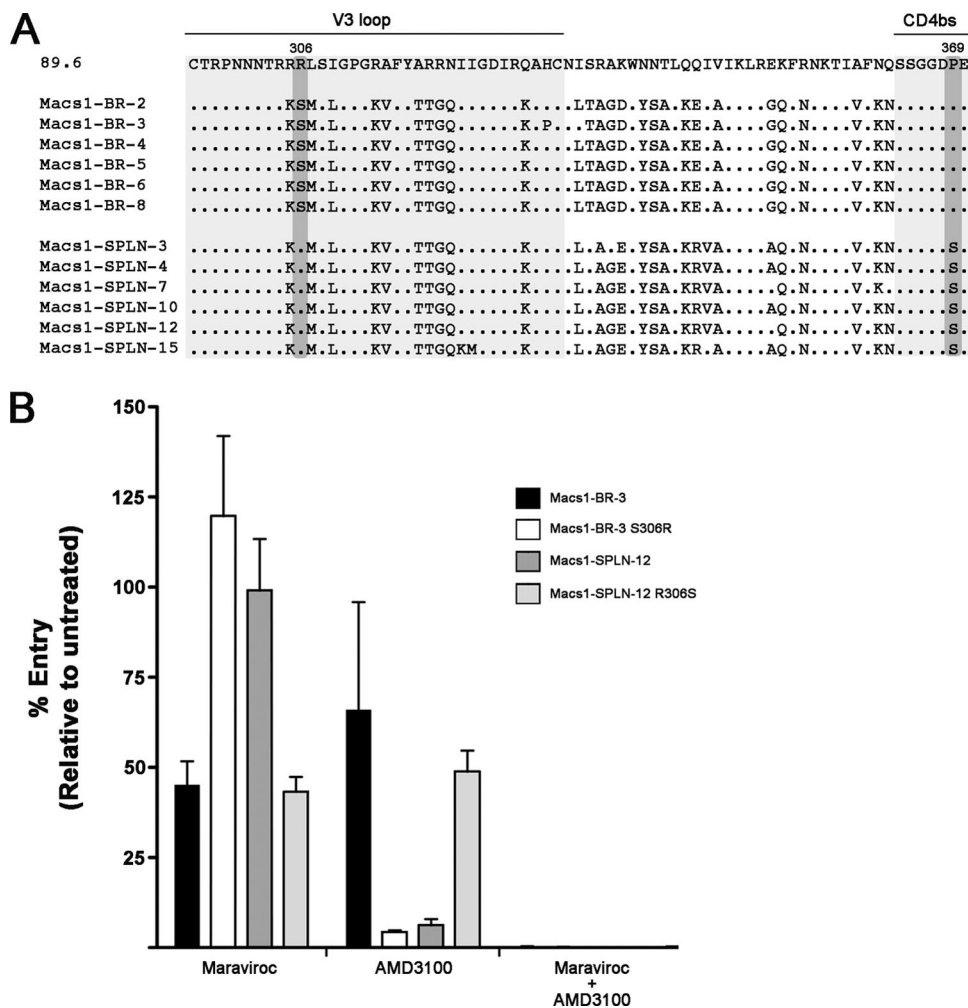


FIG. 4. Analysis of Env determinants influencing coreceptor preference. Amino acid sequences of gp120 were obtained from each of the Macs1-BR and Macs1-SPLN Env clones, as described in Materials and Methods. (A) amino acid alignments of residues 296 to 370 (relative to the numbering of amino acids in HXB2 Env) from Macs1-BR and Macs1-SPLN Envs compared to 89.6 Env. The dots indicate residues identical to those of 89.6 Env. Amino acids 306 and 369 in the V3 loop and CD4bs, which are the only amino acid changes differentiating all Macs1-BR from all Macs1-SPLN Envs, are highlighted. (B) JC53 cells were treated with maraviroc (1  $\mu$ M), AMD3100 (1  $\mu$ M), or both inhibitors prior to infection with equivalent infectious units of luciferase reporter virus pseudotyped with Macs1-BR-3, Macs1-BR-3 S306R, Macs1-SPLN-12, or Macs1-SPLN-12 R306S Env, as described in Materials and Methods. Untreated cells contained no inhibitor. HIV-1 entry was measured by luciferase activity in cell lysates and expressed as a percentage of entry in untreated cells. The data are expressed as mean values from triplicate infections, and the error bars represent standard deviations. The results are representative of three independent experiments.

brain-derived R5X4 Envs (or lack of a basic residue at this position) permits HIV-1 entry into both primary cell types via CCR5.

DISCUSSION

In this study, we showed that R5X4 Envs isolated from the brains of two individuals with AIDS had enhanced ability to use CCR5 associated with reduced dependence on residues in the CCR5 N terminus and ECL1 and ECL3 regions, whereas R5X4 Envs isolated from the spleen or blood of the same individuals had enhanced ability to use CXCR4 associated with reduced dependence on the CXCR4 N terminus and specific residues in the ECL2 region. These data were generated using R5X4 Envs from two of the three published cases of brain-derived R5X4 HIV-1 infection (30, 33, 48, 50) and from all cases where functional R5X4 Envs from paired brain and lymphoid

tissue viruses were available, and they provide evidence that brain- and lymphoid tissue-derived R5X4 Envs exist as hair trigger conformations (54) that do not require strong interactions with multiple domains of CCR5 or CXCR4, respectively, for HIV-1 entry. Infection of perivascular macrophages and microglia in the brain is mediated principally via CCR5. Importantly, we showed that brain-derived R5X4 Envs can use CCR5 for HIV-1 entry into primary cells including MDM, whereas R5X4 Envs from matched lymphoid tissue are CXCR4 restricted. Furthermore, the brain-derived R5X4 Envs had reduced dependence on the CCR5 N terminus, which, in derivatives of the R5 HIV-1 strain JRCSF (53) and primary R5 viruses isolated from late stages of HIV-1 infection (40) that have similar phenotypes, is due to increased affinity for the CCR5 ECL2 region, which results in increased efficiency of CCR5 usage. Therefore, our results support a model in which



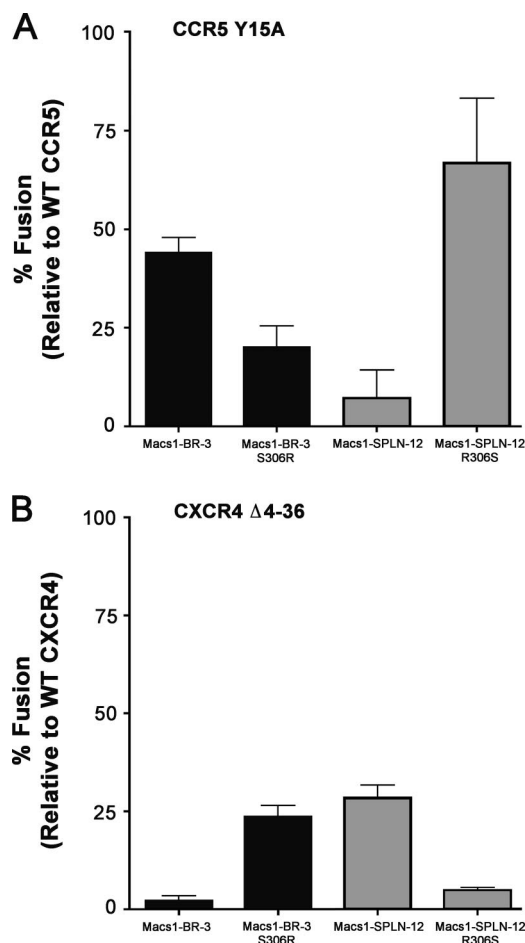


FIG. 5. Env V3 determinants influencing dependence on the CCR5 and CXCR4 N termini. Fusion assays were performed using 293T effector cells expressing Macs1-BR-3, Macs1-BR-3 S306R, Macs1-SPLN-12, or Macs1-SPLN-12 R306S Env and Cf2-Luc target cells expressing CD4 and either wild-type (WT) CCR5 or the CCR5 Y15A mutant (A) or either wild-type CXCR4 or the CXCR4  $\Delta$ 4-36 mutant (B), as described in Materials and Methods. After the background was subtracted, luciferase activity in fusion assays with mutant coreceptors was expressed as a percentage of that in fusion assays with wild-type coreceptor. The data are expressed as mean values from duplicate wells, and the error bars represent standard deviations. The results are representative of three independent experiments.

the persistence of R5X4 HIV-1 strains in the brain is mediated through alterations in the Env that increase the efficiency of CCR5 usage for HIV-1 entry, augmenting their ability to use CCR5 for entry into macrophage lineage cells. However, this study is inherently limited by the small number of brain-derived R5X4 viruses presently available. The identification and characterization of additional R5X4 viruses derived from the brain are required to fully understand the mechanisms contributing to R5X4 HIV-1 neurotropism.

The molecular mechanisms underlying the divergent patterns of coreceptor usage by brain- and lymphoid tissue-derived R5X4 Envs were studied using Macs1-BR and Macs1-SPLN Envs. The presence of S306 at position 11 in the V3 loop region of gp120 enabled brain-derived R5X4 Envs to use CCR5 preferentially for HIV-1 entry into the JC53 cell line

and to use CCR5 for HIV-1 entry into PBMC and MDM. In contrast, the presence of R306 in spleen-derived R5X4 Envs caused a CXCR4-restricted phenotype in all cell types examined. Studies characterizing the influence of V3 loop amino acid variants on the mechanism of coreceptor usage showed that S306 or R306 in brain- and spleen-derived R5X4 Envs, respectively, reduced dependence on residues in the coreceptor N terminus, but not on residues in other ECL regions that influenced coreceptor function. Current models of gp120-coreceptor interactions suggest initial binding between the V3 loop of gp120 and the coreceptor ECL2 region and subsequent interactions between the gp120 bridging sheet and the coreceptor N terminus (4, 8, 21, 38). However, the degree of dependence for the coreceptor N terminus is related to the strength of the interaction between V3 and the coreceptor ECL2 region (43, 53), suggesting a degree of functional redundancy for interaction with the N terminus by Envs with enhanced coreceptor usage. Thus, amino acid changes in V3 that differentiate brain- from spleen-derived R5X4 Envs enhance the interaction between gp120 and CCR5 or CXCR4, respectively. These data suggest distinct mechanisms of R5X4 HIV-1 persistence in different tissue compartments involving changes in the V3 loop of gp120 that enhance the ability of brain-derived R5X4 strains to use CCR5 for entry into macrophage lineage cells and that of spleen-derived R5X4 strains to use CXCR4 for entry into CD4<sup>+</sup> T cells.

Although functionally dual tropic, Macs1-BR and aBR01 Envs have gp120 sequences that are typical of R5 viruses, principally because these Envs lack basic residues at positions 11 and 25 in the gp120 V3 loop and are scored as R5 Envs by bioinformatics algorithms (48). Other functionally dual-tropic Envs derived from blood, including those from subjects C2 and DR (34), are also scored as R5 Envs by bioinformatics algorithms (48). The database sequence sets used to validate these algorithms are heavily dependent on R5 sequences, and they have recently been shown to underestimate the frequency of CXCR4 usage by R5X4 HIV-1 strains isolated from the brain and other tissues (46, 48). These results underscore the importance of phenotypic assays to accurately determine the coreceptor usage of HIV-1. In addition, since many previous studies have characterized brain-derived HIV-1 as R5 or macrophage tropic by V3 sequence without using traditional phenotypic assays (10, 42, 47, 56, 59–61, 64), the results of our study suggest the frequency of R5X4 viruses in the brain may be underestimated.

These observations have significance for understanding HIV-1 pathogenesis and for the development of antiviral therapies. Neurotropic R5X4 viruses in the brain may have a different mechanism of neurotoxicity than neurotropic R5 viruses. While neurotoxicity by R5 viruses is indirect through the release of neurotoxic viral proteins, cytokines, and metabolites from infected or activated macrophages (29), R5X4 viruses in the brain may directly cause damage to neurons. Although neurons do not express CD4 and are not infected by HIV-1, a subpopulation expresses cell surface CXCR4 (24, 25). Soluble or virion-associated gp120 from R5X4 or X4 viruses can bind to CXCR4 expressed on neurons in the absence of CD4 and thereby induce signaling and apoptosis (41, 49, 75). These results are consistent with our previous studies of the Macs1-BR and Macs1-SPLN primary isolates, which showed



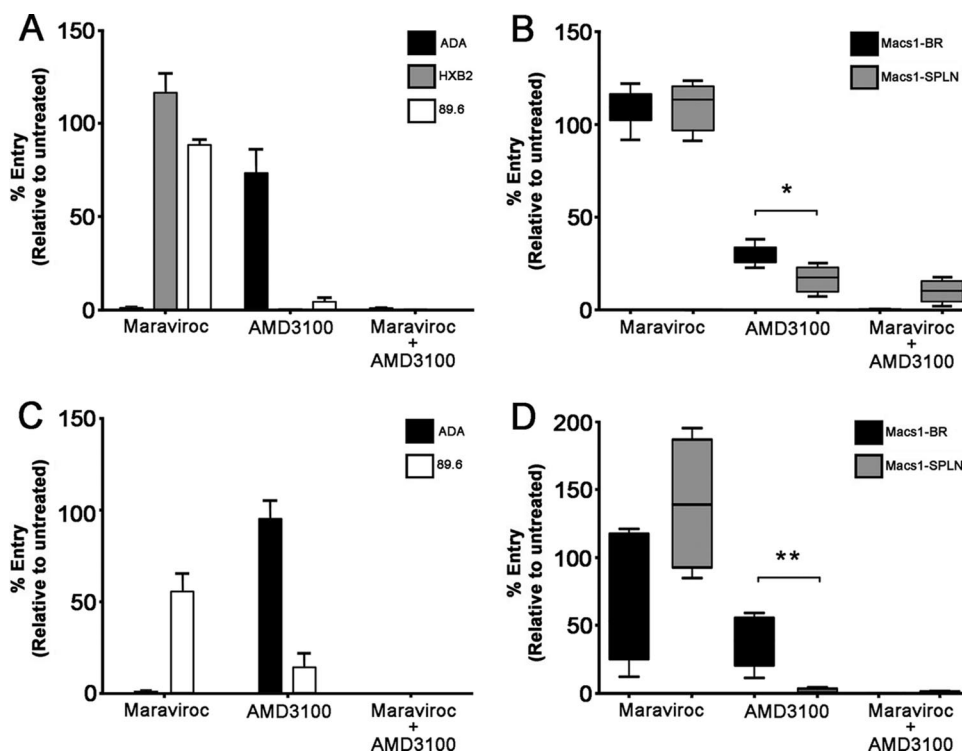


FIG. 6. Coreceptor usage for HIV-1 entry into PBMC and MDM. PBMC (A and B) or MDM (C and D) were treated with maraviroc (1  $\mu$ M), AMD3100 (1  $\mu$ M), or both inhibitors prior to infection with equivalent infectious units of Env-pseudotyped luciferase reporter virus, as described in Materials and Methods. Untreated cells contained no inhibitor. HIV-1 entry was measured by luciferase activity in cell lysates and expressed as a percentage of entry in untreated cells. The overall levels of HIV-1 entry in untreated PBMC or MDM by the brain- and spleen-derived Envs were comparable and were at least 500-fold greater than background levels (data not shown). (A and C) Inhibition of HIV-1 entry in cells inoculated with luciferase reporter virus pseudotyped with ADA, HXB2, or 89.6 Env. The data are expressed as mean values from triplicate infections, and the error bars represent standard deviations. (B and D) Inhibition of HIV-1 entry in cells inoculated with luciferase reporter virus pseudotyped with Macs1-BR Envs or Macs1-SPLN Envs. Box plots were constructed from mean values of triplicate infections with each virus using Prism version 5.0a (GraphPad Software, San Diego, CA.). The boxes represent upper and lower quartiles and median scores, and the whiskers represent minimum and maximum values. The data are expressed as mean values from triplicate infections, and the error bars represent standard deviations. The results are representative of three (PBMC) or four (MDM) independent experiments with cells obtained from different donors. *P* values were calculated using a nonparametric Mann-Whitney U test; \*, *P* < 0.05; \*\*, *P* < 0.01.

that these viruses induced greater levels of neuronal apoptosis in primary fetal brain cultures than R5 viruses isolated from the brain (30, 33). Thus, direct interactions between R5X4 HIV-1 and CXCR4 expressed on neurons may induce neuronal apoptosis or dysfunction. Since neuroactive highly active antiretroviral therapy presently offers only partial clinical benefit to patients with HAD (9, 45), the development of adjunctive therapies is being explored to reduce neurological injury during HIV-1 infection (51). Our results suggest that approaches to prevent neurological injury with adjunctive therapies should accommodate direct, as well as indirect, mechanisms of HIV-1 neurotoxicity. Furthermore, characterizing the HIV-1 phenotype in CSF, for example, using the Trofile assay (Monogram Biosciences), which is commonly used to determine HIV-1 coreceptor usage in plasma (3), may assist in deciding optimized treatment regimens for subjects with HAD.

The CCR5 inhibitor maraviroc was recently approved for use as an HIV-1 antiretroviral in treatment-experienced adults with demonstrable circulating R5 HIV-1 (22, 35). Another CCR5 inhibitor with a similar mechanism of action, vicriviroc, has recently completed phase II clinical testing in treatment-experienced and -naïve subjects (36, 44). Studies of rats

showed that maraviroc has poor penetration into the CNS, suggesting the therapeutic index of maraviroc may be insufficient to mediate antiviral effects in the brain (69). The ability of vicriviroc to penetrate the CNS has not been firmly established, but preliminary studies of rats suggest vicriviroc may penetrate the CNS better than maraviroc (66), and there are important pharmacologic differences between maraviroc and vicriviroc that suggest that vicriviroc may be better able to achieve therapeutic concentrations in the CNS of humans. Maraviroc is a substrate for the P glycoprotein drug transporter, whereas vicriviroc is not, and maraviroc is less lipophilic than vicriviroc (58, 68, 69). Further studies are required to determine the pharmacokinetics of both CCR5 inhibitors for CNS penetration in humans. However, should CCR5 inhibitors reach therapeutic concentrations in the human brain or subtherapeutic concentrations high enough to potentially promote drug resistance or selection of drug-insensitive HIV-1 variants, then the brain as a potential reservoir of R5X4 HIV-1 may need to be considered when using CCR5 inhibitors to treat HIV-1 infection.

Our studies identified the mechanisms underlying R5X4 HIV-1 persistence in different tissue reservoirs. Tissue-specific

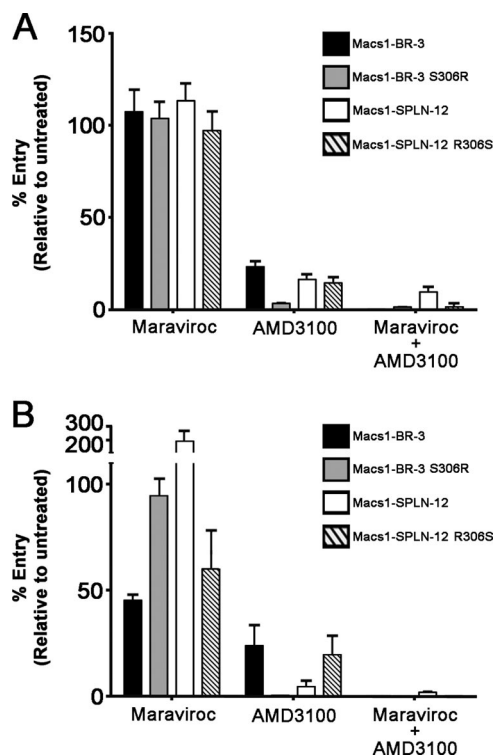


FIG. 7. Env V3 determinants influencing coreceptor usage for HIV-1 entry into PBMC and MDM. PBMC (A) or MDM (B) were treated with maraviroc (1  $\mu$ M), AMD3100 (1  $\mu$ M), or both inhibitors prior to infection with equivalent infectious units of Env-pseudotyped luciferase reporter virus, as described in Materials and Methods. Untreated cells contained no inhibitor. HIV-1 entry was measured by luciferase activity in cell lysates and expressed as a percentage of entry in untreated cells. Shown is the inhibition of HIV-1 entry in PBMC (A) or MDM (B) inoculated with luciferase reporter virus pseudotyped with Macs1-BR-3, Macs1-BR-3 S306R, Macs1-SPLN-12, or Macs1-SPLN-12 R306S Envs. The data are expressed as mean values from triplicate infections, and the error bars represent standard deviations. The results are representative of three (PBMC) or four (MDM) independent experiments with cells obtained from different donors.

changes in the V3 region of gp120 that increase the efficiency of CCR5 or CXCR4 usage and thus influence coreceptor preference may enhance the tropism of R5X4 HIV-1 strains for CCR5-expressing macrophage lineage cells in the brain and CXCR4-expressing T cells in lymphoid tissues, respectively. Understanding the molecular determinants governing the persistence of R5X4 HIV-1 in different tissue reservoirs may advance strategies to eradicate virus from protected sanctuary sites, such as the CNS, and the development of adjunctive therapies to prevent neurological injury in HAD.

#### ACKNOWLEDGMENTS

We thank J. Sodroski and B. Etemad-Gilbertson for providing Cf2-CD4/CCR5 and Cf2-Luc cells; J. Sodroski for providing pSVIII-HXB2 Env, pcDNA3-CD4, pcDNA3-CCR5, pCMV $\Delta$ P1 $\Delta$ envpA, and pHIV-1Luc plasmids; H. Göttinger for providing the pSVL-Tat plasmid; D. Kabat for providing JC53 cells; J. Sodroski, R. Doms, and M. Alizon for CCR5 and CXCR4 mutants; and H. Drummer for helpful discussions. Maraviroc was provided by Pfizer. Bicyclam JM-2987 (hydrobromide salt of AMD-3100) was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

This study was supported in part by project grants from the Australian National Health and Medical Research Council (NHMRC) to P.R.G. (433915) and M.J.C. (433920) and a multicenter program grant from the Australian NHMRC to S.W., A.L.C., and B.J.B. (358399). D.G. was supported by NIH MH83588. J.S. and L.G. were supported by Australian NHMRC Dora Lush Biomedical Research Scholarships. P.R.G. is the recipient of an Australian NHMRC R. Douglas Wright Biomedical Career Development Award.

#### REFERENCES

- Alkhatib, G., C. Combadiere, C. C. Broder, Y. Feng, P. E. Kennedy, P. M. Murphy, and E. A. Berger. 1996. CC CKR5: a RANTES, MIP-1 $\alpha$ , MIP-1 $\beta$  receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* **272**: 1955–1958.
- Blankson, J. N., D. Persaud, and R. F. Siliciano. 2002. The challenge of viral reservoirs in HIV-1 infection. *Annu. Rev. Med.* **53**:557–593.
- Braun, P., and F. Wiesmann. 2007. Phenotypic assays for the determination of coreceptor tropism in HIV-1 infected individuals. *Eur. J. Med. Res.* **12**:463–472.
- Brelot, A., N. Heveker, K. Adema, M. J. Hosie, B. Willett, and M. Alizon. 1999. Effect of mutations in the second extracellular loop of CXCR4 on its utilization by human and feline immunodeficiency viruses. *J. Virol.* **73**:2576–2586.
- Cardozo, T., T. Kimura, S. Philpott, B. Weiser, H. Burger, and S. Zolla-Pazner. 2007. Structural basis for coreceptor selectivity by the HIV type 1 V3 loop. *AIDS Res. Hum. Retrovir.* **23**:415–426.
- Choe, H., M. Farzan, Y. Sun, N. Sullivan, B. Rollins, P. D. Ponath, L. Wu, C. R. Mackay, G. LaRosa, W. Newman, N. Gerard, C. Gerard, and J. Sodroski. 1996. The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* **85**:1135–1148.
- Collman, R., J. W. Balliet, S. A. Gregory, H. Friedman, D. L. Kolson, N. Nathanson, and A. Srinivasan. 1992. An infectious molecular clone of an unusual macrophage-tropic and highly cytopathic strain of human immunodeficiency virus type 1. *J. Virol.* **66**:7517–7521.
- Cormier, E. G., and T. Dragic. 2002. The crown and stem of the V3 loop play distinct roles in human immunodeficiency virus type 1 envelope glycoprotein interactions with the CCR5 coreceptor. *J. Virol.* **76**:8953–8957.
- Cysique, L. A., P. Maruff, and B. J. Brew. 2004. Antiretroviral therapy in HIV infection: are neurologically active drugs important? *Arch. Neurol.* **61**:1699–1704.
- Di Stefano, M., S. Wilt, F. Gray, M. Dubois-Dalcq, and F. Chiodi. 1996. HIV type 1 V3 sequences and the development of dementia during AIDS. *AIDS Res. Hum. Retrovir.* **12**:471–476.
- Doms, R. W. 2000. Beyond receptor expression: the influence of receptor conformation, density, and affinity in HIV-1 infection. *Virology* **276**:229–237.
- Doms, R. W., and D. Trono. 2000. The plasma membrane as a combat zone in the HIV battlefield. *Genes Dev.* **14**:2677–2688.
- Doranz, B. J., Z. H. Lu, J. Rucker, T. Y. Zhang, M. Sharron, Y. H. Cen, Z. X. Wang, H. H. Guo, J. G. Du, M. A. Accavitti, R. W. Doms, and S. C. Peiper. 1997. Two distinct CCR5 domains can mediate coreceptor usage by human immunodeficiency virus type 1. *J. Virol.* **71**:6305–6314.
- Doranz, B. J., M. J. Orsini, J. D. Turner, T. L. Hoffman, J. F. Berson, J. A. Hoxie, S. C. Peiper, L. F. Brass, and R. W. Doms. 1999. Identification of CXCR4 domains that support coreceptor and chemokine receptor functions. *J. Virol.* **73**:2752–2761.
- Dragic, T., V. Litwin, G. P. Allaway, S. R. Martin, Y. Huang, K. A. Nagashima, C. Cayanan, P. J. Maddon, R. A. Koup, J. P. Moore, and W. A. Paxton. 1996. HIV-1 entry into CD4<sup>+</sup> cells is mediated by the chemokine receptor CC-CCR-5. *Nature* **381**:667–673.
- Dunfee, R., E. Thomas, P. R. Gorry, J. Wang, P. Ancuta, and D. Gabuzda. 2006. Mechanisms of HIV-1 neurotropism. *Curr. HIV Res.* **4**:267–278.
- Dunfee, R. L., E. R. Thomas, P. R. Gorry, J. Wang, J. Taylor, K. Kunstan, S. M. Wolinsky, and D. Gabuzda. 2006. The HIV Env variant N283 enhances macrophage tropism and is associated with brain infection and dementia. *Proc. Natl. Acad. Sci. USA* **103**:15160–15165.
- Ellery, P. J., E. Tippet, Y. L. Chiu, G. Paukovics, P. U. Cameron, A. Solomon, S. R. Lewin, P. R. Gorry, A. Jaworski, W. C. Greene, S. Sonza, and S. M. Crowe. 2007. The CD16<sup>+</sup> monocyte subset is more permissive to infection and preferentially harbors HIV-1 in vivo. *J. Immunol.* **178**:6581–6589.
- Etemad-Moghadam, B., Y. Sun, E. K. Nicholson, M. Fernandes, K. Liou, R. Gomila, J. Lee, and J. Sodroski. 2000. Envelope glycoprotein determinants of increased fusogenicity in a pathogenic simian-human immunodeficiency virus (SHIV-KB9) passaged in vivo. *J. Virol.* **74**:4433–4440.
- Farzan, M., H. Choe, L. Vaca, K. Martin, Y. Sun, E. Desjardins, N. Ruffing, L. Wu, R. Wyatt, N. Gerard, C. Gerard, and J. Sodroski. 1998. A tyrosine-rich region in the N terminus of CCR5 is important for human immunodeficiency virus type 1 entry and mediates an association between gp120 and CCR5. *J. Virol.* **72**:1160–1164.
- Farzan, M., T. Mirzabekov, P. Kolchinsky, R. Wyatt, M. Cayabyab, N. P.

- Gerard, C. Gerard, J. Sodroski, and H. Choe. 1999. Tyrosine sulfation of the amino terminus of CCR5 facilitates HIV-1 entry. *Cell* **96**:667–676.
22. Fatkenheuer, G., M. Nelson, A. Lazzarin, I. Konourina, A. I. Hoepelman, H. Lampiris, B. Hirschel, P. Tebas, F. Raffi, B. Trottier, N. Bellos, M. Saag, D. A. Cooper, M. Westby, M. Tawadrous, J. F. Sullivan, C. Ridgway, M. W. Dunne, S. Felstead, H. Mayer, and E. van der Ryst. 2008. Subgroup analyses of maraviroc in previously treated R5 HIV-1 infection. *N. Engl. J. Med.* **359**:1442–1455.
23. Feng, Y., C. C. Broder, P. E. Kennedy, and E. A. Berger. 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272**:872–877.
24. Gabuzda, D., and J. Wang. 2000. Chemokine receptors and mechanisms of cell death in HIV neuropathogenesis. *J. Neurovirol.* **6**(Suppl. 1):S24–S32.
25. Gabuzda, D., and J. Wang. 1999. Chemokine receptors and virus entry in the central nervous system. *J. Neurovirol.* **5**:643–658.
26. Gao, F., S. G. Morrison, D. L. Robertson, C. L. Thornton, S. Craig, G. Karlsson, J. Sodroski, M. Morgado, B. Galvao-Castro, H. von Briesen, et al. 1996. Molecular cloning and analysis of functional envelope genes from human immunodeficiency virus type 1 sequence subtypes A through G. *J. Virol.* **70**:1651–1667.
27. Gartner, S., P. Markovits, D. M. Markovitz, R. F. Betts, and M. Popovic. 1986. Virus isolation from and identification of HTLV-III/LAV-producing cells in brain tissue from a patient with AIDS. *JAMA* **256**:2365–2371.
28. Gartner, S., P. Markovits, D. M. Markovitz, M. H. Kaplan, R. C. Gallo, and M. Popovic. 1986. The role of mononuclear phagocytes in HTLV-III/LAV infection. *Science* **233**:215–219.
29. Gonzalez-Scarano, F., and J. Martin-Garcia. 2005. The neuropathogenesis of AIDS. *Nat. Rev. Immunol.* **5**:69–81.
30. Gorry, P. R., G. Bristol, J. A. Zack, K. Ritola, R. Swanstrom, C. J. Birch, J. E. Bell, N. Bannett, K. Crawford, H. Wang, D. Schols, E. De Clercq, K. Kunstman, S. M. Wolinsky, and D. Gabuzda. 2001. Macrophage tropism of human immunodeficiency virus type 1 isolates from brain and lymphoid tissues predicts neurotropism independent of coreceptor specificity. *J. Virol.* **75**:10073–10089.
31. Gorry, P. R., M. Churchill, S. M. Crowe, A. L. Cunningham, and D. Gabuzda. 2005. Pathogenesis of macrophage tropic HIV. *Curr. HIV Res.* **3**:53–60.
32. Gorry, P. R., R. L. Dunfee, M. E. Mefford, K. Kunstman, T. Morgan, J. P. Moore, J. R. Mascola, K. Agopian, G. H. Holm, A. Mehle, J. Taylor, M. Farzan, H. Wang, P. Ellery, S. J. Willey, P. R. Clapham, S. M. Wolinsky, S. M. Crowe, and D. Gabuzda. 2007. Changes in the V3 region of gp120 contribute to unusually broad coreceptor usage of an HIV-1 isolate from a CCR5  $\Delta 32$  heterozygote. *Virology* **362**:163–178.
33. Gorry, P. R., J. Taylor, G. H. Holm, A. Mehle, T. Morgan, M. Cayabyab, M. Farzan, H. Wang, J. E. Bell, K. Kunstman, J. P. Moore, S. M. Wolinsky, and D. Gabuzda. 2002. Increased CCR5 affinity and reduced CCR5/CD4 dependence of a neurovirulent primary human immunodeficiency virus type 1 isolate. *J. Virol.* **76**:6277–6292.
34. Gray, L., M. J. Churchill, N. Keane, J. Sterjovski, A. M. Ellett, D. F. J. Purcell, P. Pombourios, C. Kol, B. Wang, N. Saksena, S. L. Wesseling, P. Price, M. French, D. Gabuzda, and P. R. Gorry. 2006. Genetic and functional analysis of R5X4 human immunodeficiency virus type 1 envelope glycoproteins derived from two individuals homozygous for the CCR5 $\Delta 32$  allele. *J. Virol.* **80**:3684–3691.
35. Gulick, R. M., J. Lalezari, J. Goodrich, N. Clumeck, E. DeJesus, A. Horban, J. Nadler, B. Clotet, A. Karlsson, M. Wohlfeiler, J. B. Montana, M. McHale, J. Sullivan, C. Ridgway, S. Felstead, M. W. Dunne, E. van der Ryst, and H. Mayer. 2008. Maraviroc for previously treated patients with R5 HIV-1 infection. *N. Engl. J. Med.* **359**:1429–1441.
36. Gulick, R. M., Z. Su, C. Flexner, M. D. Hughes, P. R. Skolnik, T. J. Wilkin, R. Gross, A. Krambrink, E. Coakley, W. L. Greaves, A. Zolopa, R. Reichman, C. Godfrey, M. Hirsch, and D. R. Kuritzkes. 2007. Phase 2 study of the safety and efficacy of vicriviroc, a CCR5 inhibitor, in HIV-1-infected, treatment-experienced patients: AIDS clinical trials group 5211. *J. Infect. Dis.* **196**:304–312.
37. Hoffman, T. L., and R. W. Doms. 1999. HIV-1 envelope determinants for cell tropism and chemokine receptor use. *Mol. Membr. Biol.* **16**:57–65.
38. Huang, C. C., M. Tang, M. Y. Zhang, S. Majeed, E. Montabana, R. L. Stanfield, D. S. Dimitrov, B. Korber, J. Sodroski, I. A. Wilson, R. Wyatt, and P. D. Kwong. 2005. Structure of a V3-containing HIV-1 gp120 core. *Science* **310**:1025–1028.
39. Jensen, M. A., F. S. Li, A. B. van 't Wout, D. C. Nickle, D. Shriner, H. X. He, S. McLaughlin, R. Shankarappa, J. B. Margolick, and J. I. Mullins. 2003. Improved coreceptor usage prediction and genotypic monitoring of R5-to-X4 transition by motif analysis of human immunodeficiency virus type 1 env V3 loop sequences. *J. Virol.* **77**:13376–13388.
40. Karlsson, L., L. Antonsson, Y. Shi, M. Oberg, A. Karlsson, J. Albert, B. Olde, C. Oman, M. Jansson, and E. M. Fenyo. 2004. Coevolution of RANTES sensitivity and mode of CCR5 receptor use by human immunodeficiency virus type 1 of the R5 phenotype. *J. Virol.* **78**:11807–11815.
41. Kaul, M., G. A. Garden, and S. A. Lipton. 2001. Pathways to neuronal injury and apoptosis in HIV-associated dementia. *Nature* **410**:988–994.
42. Korber, B. T., K. J. Kunstman, B. K. Patterson, M. Furtado, M. M. McEvilly, R. Levy, and S. M. Wolinsky. 1994. Genetic differences between blood- and brain-derived viral sequences from human immunodeficiency virus type 1-infected patients: evidence of conserved elements in the V3 region of the envelope protein of brain-derived sequences. *J. Virol.* **68**:7467–7481.
43. Laakso, M. M., F. H. Lee, B. Haggarty, C. Agrawal, K. M. Nolan, M. Biscone, J. Romano, A. P. Jordan, G. J. Leslie, E. G. Meissner, L. Su, J. A. Hoxie, and R. W. Doms. 2007. V3 loop truncations in HIV-1 envelope impart resistance to coreceptor inhibitors and enhanced sensitivity to neutralizing antibodies. *PLoS Pathog.* **3**:e117.
44. Landovitz, R. J., J. B. Angel, C. Hoffmann, H. Horst, M. Opravil, J. Long, W. Greaves, and G. Fatkenheuer. 2008. Phase II study of vicriviroc versus efavirenz (both with zidovudine/lamivudine) in treatment-naïve subjects with HIV-1 infection. *J. Infect. Dis.* **198**:1113–1122.
45. Letendre, S., J. Marquie-Beck, E. Capparelli, B. Best, D. Clifford, A. C. Collier, B. Gelman, J. C. McArthur, J. A. McCutchan, S. Morgello, D. Simpson, I. Grant, and R. J. Ellis. 2008. Validation of the CNS penetration-effectiveness rank for quantifying antiretroviral penetration into the central nervous system. *Arch. Neurol.* **65**:65–70.
46. Low, A. J., W. Dong, D. Chan, T. Sing, R. Swanstrom, M. Jensen, S. Pillai, B. Good, and P. R. Harrigan. 2007. Current V3 genotyping algorithms are inadequate for predicting X4 co-receptor usage in clinical isolates. *AIDS* **21**:F17–F24.
47. McGavin, C. H., S. A. Land, K. L. Seibire, D. J. Hooker, A. D. Gurusinge, and C. J. Birch. 1996. Syncytium-inducing phenotype and zidovudine susceptibility of HIV-1 isolated from post-mortem tissue. *AIDS* **10**:47–53.
48. Mefford, M. E., P. R. Gorry, K. Kunstman, S. M. Wolinsky, and D. Gabuzda. 2008. Bioinformatic prediction programs underestimate the frequency of CXCR4 usage by R5X4 HIV type 1 in brain and other tissues. *AIDS Res. Hum. Retrovir.* **24**:1215–1220.
49. Meucci, O., A. Fatatis, A. A. Simen, T. J. Bushell, P. W. Gray, and R. J. Miller. 1998. Chemokines regulate hippocampal neuronal signaling and gp120 neurotoxicity. *Proc. Natl. Acad. Sci. USA* **95**:14500–14505.
50. Ohagen, A., A. Devitt, K. J. Kunstman, P. R. Gorry, P. P. Rose, B. Korber, J. Taylor, R. Levy, R. L. Murphy, S. M. Wolinsky, and D. Gabuzda. 2003. Genetic and functional analysis of full-length human immunodeficiency virus type 1 env genes derived from brain and blood of patients with AIDS. *J. Virol.* **77**:12336–12345.
51. Perry, S. W., J. P. Norman, and H. A. Gelbard. 2005. Adjunctive therapies for HIV-1 associated neurologic disease. *Neurotox. Res.* **8**:161–166.
52. Pierson, T., J. McArthur, and R. F. Siliciano. 2000. Reservoirs for HIV-1: mechanisms for viral persistence in the presence of antiviral immune responses and antiretroviral therapy. *Annu. Rev. Immunol.* **18**:665–708.
53. Platt, E. J., S. E. Kuhmann, P. P. Rose, and D. Kabat. 2001. Adaptive mutations in the V3 loop of gp120 enhance fusogenicity of human immunodeficiency virus type 1 and enable use of a CCR5 coreceptor that lacks the amino-terminal sulfated region. *J. Virol.* **75**:12266–12278.
54. Platt, E. J., D. M. Shea, P. P. Rose, and D. Kabat. 2005. Variants of human immunodeficiency virus type 1 that efficiently use CCR5 lacking the tyrosine-sulfated amino terminus have adaptive mutations in gp120, including loss of a functional N-glycan. *J. Virol.* **79**:4357–4368.
55. Platt, E. J., K. Wehrly, S. E. Kuhmann, B. Chesebro, and D. Kabat. 1998. Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. *J. Virol.* **72**:2855–2864.
56. Reddy, R. T., C. L. Achim, D. A. Sirko, S. Tehrani, F. G. Kraus, F. Wong-Staal, and C. A. Wiley. 1996. Sequence analysis of the V3 loop in brain and spleen of patients with HIV encephalitis. *AIDS Res. Hum. Retrovir.* **12**:477–482.
57. Resch, W., N. Hoffman, and R. Swanstrom. 2001. Improved success of phenotype prediction of the human immunodeficiency virus type 1 from envelope variable loop 3 sequence using neural networks. *Virology* **288**:51–62.
58. Schurmann, D., G. Fatkenheuer, J. Reynes, C. Michelet, F. Raffi, J. van Lier, M. Caceres, A. Keung, A. Sansone-Parsons, L. M. Dunkle, and C. Hoffmann. 2007. Antiviral activity, pharmacokinetics and safety of vicriviroc, an oral CCR5 antagonist, during 14-day monotherapy in HIV-infected adults. *AIDS* **21**:1293–1299.
59. Sharpless, N. E., W. A. O'Brien, E. Verdin, C. V. Kufta, I. S. Chen, and M. Dubois-Dalq. 1992. Human immunodeficiency virus type 1 tropism for brain microglial cells is determined by a region of the env glycoprotein that also controls macrophage tropism. *J. Virol.* **66**:2588–2593.
60. Shimizu, N., Y. Haraguchi, Y. Takeuchi, Y. Soda, K. Kanbe, and H. Hoshino. 1999. Changes in and discrepancies between cell tropisms and coreceptor uses of human immunodeficiency virus type 1 induced by single point mutations at the V3 tip of the env protein. *Virology* **259**:324–333.
61. Shimizu, N. S., N. G. Shimizu, Y. Takeuchi, and H. Hoshino. 1994. Isolation and characterization of human immunodeficiency virus type 1 variants infectious to brain-derived cells: detection of common point mutations in the V3 region of the env gene of the variants. *J. Virol.* **68**:6130–6135.
62. Siliciano, J. D., J. Kajdas, D. Finzi, T. C. Quinn, K. Chadwick, J. B. Mar-



- golick, C. Kovacs, S. J. Gange, and R. F. Siliciano. 2003. Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4<sup>+</sup> T cells. *Nat. Med.* **9**:727–728.
63. Siliciano, R. F. 1999. Latency and reservoirs for HIV-1. *AIDS* **13**(Suppl. A):S49–S58.
  64. Smit, T. K., B. Wang, T. Ng, R. Osborne, B. Brew, and N. K. Saksena. 2001. Varied tropism of HIV-1 isolates derived from different regions of adult brain cortex discriminate between patients with and without AIDS dementia complex (ADC): evidence for neurotropic HIV variants. *Virology* **279**:509–526.
  65. Sterjovski, J., M. J. Churchill, A. Ellett, L. R. Gray, M. J. Roche, R. L. Dunfee, D. F. Purcell, N. Saksena, B. Wang, S. Sonza, S. L. Wesselingh, I. Karlsson, E. M. Fenyo, D. Gabuzda, A. L. Cunningham, and P. R. Gorry. 2007. Asn 362 in gp120 contributes to enhanced fusogenicity by CCR5-restricted HIV-1 envelope glycoprotein variants from patients with AIDS. *Retrovirology* **4**:89.
  66. Tagat, J. R., S. W. McCombie, D. Nazareno, M. A. Labroli, Y. Xiao, R. W. Steensma, J. M. Strizki, B. M. Baroudy, K. Cox, J. Lachowicz, G. Varty, and R. Watkins. 2004. Piperazine-based CCR5 antagonists as HIV-1 inhibitors. IV. Discovery of 1-[(4,6-dimethyl-5-pyrimidinyl)carbonyl]-4-[4-[2-methoxy-1(*R*)-4-(trifluoromethyl)phenyl]ethyl-3(*S*)-methyl-1-piperazinyl]-4-methylpiperidine (Sch-417690/Sch-D), a potent, highly selective, and orally bioavailable CCR5 antagonist. *J. Med. Chem.* **47**:2405–2408.
  67. Thomas, E. R., R. L. Dunfee, J. Stanton, D. Bogdan, J. Taylor, K. Kunstman, J. E. Bell, S. M. Wolinsky, and D. Gabuzda. 2007. Macrophage entry mediated by HIV Envs from brain and lymphoid tissues is determined by the capacity to use low CD4 levels and overall efficiency of fusion. *Virology* **360**:105–119.
  68. Walker, D. K., S. Abel, P. Comby, G. J. Muirhead, A. N. Nedderman, and D. A. Smith. 2005. Species differences in the disposition of the CCR5 antagonist, UK-427,857, a new potential treatment for HIV. *Drug Metab. Dispos.* **33**:587–595.
  69. Walker, D. K., S. J. Bowers, R. J. Mitchell, M. J. Potchoiba, C. M. Schroeder, and H. F. Small. 2008. Preclinical assessment of the distribution of maraviroc to potential human immunodeficiency virus (HIV) sanctuary sites in the central nervous system (CNS) and gut-associated lymphoid tissue (GALT). *Xenobiotica* **38**:1330–1339.
  70. Xu, S., X. Huang, H. Xu, and C. Zhang. 2007. Improved prediction of coreceptor usage and phenotype of HIV-1 based on combined features of V3 loop sequence using random forest. *J. Microbiol.* **45**:441–446.
  71. Yang, X., V. Tomov, S. Kurteva, L. Wang, X. Ren, M. K. Gorny, S. Zolla-Pazner, and J. Sodroski. 2004. Characterization of the outer domain of the gp120 glycoprotein from human immunodeficiency virus type 1. *J. Virol.* **78**:12975–12986.
  72. Yang, X., R. Wyatt, and J. Sodroski. 2001. Improved elicitation of neutralizing antibodies against primary human immunodeficiency viruses by soluble stabilized envelope glycoprotein trimers. *J. Virol.* **75**:1165–1171.
  73. Yi, Y., S. N. Isaacs, D. A. Williams, I. Frank, D. Schols, E. De Clercq, D. L. Kolson, and R. G. Collman. 1999. Role of CXCR4 in cell-cell fusion and infection of monocyte-derived macrophages by primary human immunodeficiency virus type 1 (HIV-1) strains: two distinct mechanisms of HIV-1 dual tropism. *J. Virol.* **73**:7117–7125.
  74. Yi, Y., F. Shaheen, and R. G. Collman. 2005. Preferential use of CXCR4 by R5X4 human immunodeficiency virus type 1 isolates for infection of primary lymphocytes. *J. Virol.* **79**:1480–1486.
  75. Zheng, J., A. Ghorpade, D. Niemann, R. L. Cotter, M. R. Thylin, L. Epstein, J. M. Swartz, R. B. Shepard, X. Liu, A. Nukuna, and H. E. Gendelman. 1999. Lymphotropic virions affect chemokine receptor-mediated neural signaling and apoptosis: implications for human immunodeficiency virus type 1-associated dementia. *J. Virol.* **73**:8256–8267.